

METHOD AND DEVICE FOR DETERMINING THE VOLUME OF A LIQUID SAMPLE

This application claims priority under 35 U.S.C. §119 to Swiss patent application 2000 2252/00, filed November 17, 2000 and Swiss patent application 2000 2281/00, filed November 23, 2000.

FIELD OF THE INVENTION

10

The present invention relates to a method for determining the volume of a sample of a liquid (A), wherein, in order to stain the liquid (A), a specific concentration of a chromophoric indicator is provided in this liquid (A), a sample is separated from the liquid (A), the optical absorp-
15 tion of the separated sample is measured, and the volume of the separated sample is determined by correlation of the measured optical absorption with the concentration of indicator in this liquid (A).

BACKGROUND OF THE INVENTION

20

It is known that droplets with a volume of more than 10 μ l can be dispensed from the air very easily, since if the pipette is correctly manipulated, the droplets leave the pipette tip of their own accord. The droplet size is then determined by the physical properties of the sample liquid,
25 such as surface tension or viscosity. The droplet size thus limits the resolution of the quantity of liquid to be dispensed.

The aspirating and dispensing, i.e. the pipetting of liquid samples with a volume of less than 10 μ l, in contrast, typically requires instruments and techniques which guarantee the dispensing of such small samples.
30 The dispensing of a liquid with a pipette tip, i.e. with the endpiece of a device for aspirating and/or dispensing sample liquid, can occur from the air ("from air") or by touching a surface. This surface can be the solid surface of a container ("on tip touch"), into which the liquid sam-

ple is to be dispensed. It can also be the surface of a liquid in this container ("on liquid surface"). A mixing procedure following the dispensing is recommended -- particularly for very small sample volumes in the nanoliter or even picoliter range -- so that uniform distribution of the sample volume in a diluent is ensured.

Systems for separating samples from a liquid are known as pipettors. Such systems serve, for example, for dispensing liquids into the wells of Standard Microtitration Plates™ (trademark of Beckman Coulter, Inc., 4300 N. Harbour Blvd., P.O. Box 3100 Fullerton, CA, USA 92834) and/or microplates with 96 wells. The reduction of the sample volumes (e.g. for filling high-density microplates with 384, 864, 1536, or even more wells) plays an increasingly important role, with the precision of the sample volume dispensed being of great importance. The elevation of the number of samples typically also requires miniaturization of the experiment, so that the use of a pipettor is necessary and special requirements must be placed on the precision of sample volumes and the accuracy of the movement control and/or of the dispensing of this pipettor.

Disposable tips significantly reduce the danger of unintentional transfer of parts of the sample (contamination). Simple disposable tips are known (so-called "air-displacement tips"), whose geometry and material is optimized for the exact aspirating and/or dispensing of very small volumes. The use of so-called "positive-displacement tips", which have a pump plunger inside, is also known.

For automation, two procedures must be differentiated from one another: the defined aspiration and the subsequent dispensing of liquid samples. Between these procedures, typically the pipette tip is moved by the experimenter or by a robot, so that the aspiration location of a liquid sample is different from its dispensing location. For the precision of dispensing and/or aspiration/dispensing, only the liquid system is

0902273.74604

essential, which comprises a pump (e.g. a diluter implemented as a syringe pump), tubing, and an endpiece (pipette tip).

5 The precision (ACC = accuracy) and reproducibility (CV = coefficient of variation) of the dispensing and/or aspiration/dispensing of a liquid sample can be influenced by greatly varying parameters. The speed of dispensing largely determines, for example, how the droplet breaks away from the pipette tip.

10 In principle, two basic modes are differentiated in pipetting: single pipetting and multipipetting. In the single pipetting mode, a liquid sample is aspirated and dispensed at another location. In the multipipetting mode, a larger volume of liquid is aspirated at one time and subsequently dispensed in several -- typically equivalent -- portions (aliquots)
15 at one or more different locations, e.g. in various wells of a Standard Microtitration Plate™.

The measurement of the volume of a liquid sample, however, does not take into consideration the way in which a droplet was separated: in
20 Europe, the norm ISO/DIS 8655-1 of the International Organization for Standardization (ISO) (whose main offices are in Geneva, Switzerland) has been available at least in draft form since 1990. This norm defines the basic conditions for performing laboratory work with dispensing devices, such as pipettes, dispensers, and burettes. Known national
25 norms, such as ASTM (USA), British Standard (GB), or the newest draft DIN 12650 (Germany), have to fit themselves into the system of the ISO norm ISO/DIS 8655-1.

The norm DIN 12650 essentially differentiates two methodical categories for testing measurement accuracy of dispensers in its 4th draft
30 from 1996. These are the gravimetric and non-gravimetric methods. Since not every laboratory has available sufficient balanced weighing stations and costly scales with the necessary resolution (six decimal

places) for performing gravimetric measurements, photometric tests for hand pipettes, e.g. for the range of sample volumes from 0.2 to 1 µl, have been offered by the industry (e.g. the firm EPPENDORF AG, Barkhausenweg 1, D-22339 Hamburg, Germany).

5

A further method is known from the article "Performance Verification of Small Volume Mechanical Action Pipettes" by Richard H. Curtis [Cal.Lab, May/June 1996]. In consideration of the difficulties (e.g. evaporation, vibrations, static charge of the samples) of the application of gravimetric methods to a liquid sample in the microliter range, in this article an integrated system was suggested based on using colorimetric substances. However, the concentration of indicator substance whose optical density is to be measured must be known exactly. This optical density is calculated as $\log_{10} (1/T)$, with T referred to as transmittance. This transmittance corresponds to I/I_0 , i.e. the ratio of output intensity and input intensity of the light beams penetrating the sample. Furthermore, the device used for measuring the optical density must also meet international norms. In addition, problems such as a dependence of the measurement on the sample temperature, the appearance of changes in the solution, and the appearance of wear in the measurement cuvette must be considered. The firm ARTEL Inc., 25 Bradley Drive Westbrook, Maine, USA, produces the "Artel PCS™ Pipette Calibration System" of this type. It essentially consists of a prefilled, sealed test glass with 4.75 ml of an exactly defined concentration of a copper chloride solution and an instrument for measuring the optical absorption (wherein optical absorption $A = I_0/I = -\log_{10} T$) of this solution at a wavelength of 730 nm. The test glass is inserted in the instrument and remains in place during the entire calibration process. The experimenter opens the seal of the test glass and adds a sample corresponding to the desired measurement precision to the glass with the pipette to be checked, and then reseals the seal. The sample added is a solution of "Ponceau S", an organic test substance, which, among other things, is selected due to its long-term stability and good "pipettability" (similar to

- water, even at high concentrations) and its wide, well-defined absorption peak at 520 nm. The absorption peaks of the copper chloride solution and of the test solution "Ponceanu S" do not overlap. In addition, the test solution contains biocides, in order to prevent the growth of
- 5 microorganisms, and a pH-stabilizing buffer. The device mixes the two solutions with an integrated mixer and determines the absorption at 520 nm (Ponceanu S) and at 730 nm (copper chloride). The volume of the sample added is then calculated on the basis of these two measured values and the known initial concentrations. Although this system has
- 10 the advantage that the measurement of the optical absorption is allowed independently of the path length and irregularities in the test glass, it nonetheless has the disadvantage that it cannot be adapted at a reasonable expense for usage in a multichannel pipetting robot.
- 15 A further calibration method of this type uses "Orange G" as the test substance, which allows an absorption measurement with high sensitivity. However, it is disadvantageous in this case that the flat molecules of this test substance have a high adhesion to the inner walls of the pipette tip and/or to the tubings, troughs, and/or wells of microplates.
- 20 Therefore, an uncontrollable reduction of the Orange G concentration in the test liquid occurs, which makes the reliability of the test questionable.

- Another method of this type is known from Belgian patent No. BE 761
- 25 537, which describes the analysis of various substances with increased precision, particularly automatic analysis, which depends on the sample volume of the substance. According to this invention, one mixes chromium in the form of $\text{Cr}_2(\text{SO}_4)_3 \cdot 10 \text{ H}_2\text{O}$ into a sample as an indicator, in order to obtain a specific concentration of chromium (III) therein. With
- 30 reference to the chromium (III) concentration measured, the effective volume of the sample is calculated. The sample volumes are in the milliliter range.

Cr₂(SO₄)₃ · 10 H₂O exists in aqueous solution as [Cr(H₂O)₆]³⁺. According to the literature (see, for example, W. Schneider in "Einführung in die Koordinationschemie", Springer Verlag Berlin, Heidelberg, New York 1968, pp. 115-117), the aqueous complex [Cr(H₂O)₆]³⁺ has a molar extinction coefficient (ϵ) of only approximately 13 (where an ϵ of less than 100 is a low to average value). In pure aqueous complexes, the extinction coefficient ϵ is approximately 50. The concentration of a pigment and the optical absorption are linked via the Beer-Lambert law

$$(A = c \cdot \epsilon \cdot l).$$

where:

A = optical absorption

c = concentration of the dissolved material [M = Mol/L]

ϵ = molar extinction coefficient of the dissolved material [1/(M·cm)]

l = layer thickness (the liquid which the light must pass through) [cm].

Due to limitations in spectrophotometric hardware reasons, the art (cf. Bruno Lange et al. in "Photometrische Analyse", VCH Verlagsgesellschaft mbH, Weinheim, 1987, p. 21) recommends that measurements only be performed in the absorption range from 0.1 to 1. The sensitivity of the measurement system increases with higher ϵ . In order to be able to measure a volume of 1 μ l in a final volume of 200 μ l with an optical absorption of 0.1, the concentration of [Cr(H₂O)₆]³⁺ must be at least 15 Mol/L according to the Beer-Lambert law. However, the physical properties of the sample are significantly changed by these high concentrations, and this, of course, is undesirable.

SUMMARY OF THE INVENTION

The object of the present invention is to provide an alternative method and a corresponding device for determining the volume of a sample of a

liquid that eliminates the inadequacies from the prior art and allows calibration even in the sub-microliter range.

The methods of the invention are provided according to the features of independent claim 1. The devices of the invention are provided according to the features of the independent claim 15. Additional and/or refining features arise from the dependent claims.

The metal complex pigments used according to the present invention have extinction coefficients ϵ of more than 10,000, which, when compared to the prior art, permits significantly more sensitive measurement systems to be used. For example, iron-tris-bathophenanthroline-disulfonic acid disodium complex $[\text{Fe}(\text{C}_{24}\text{H}_{16}\text{N}_2\text{O}_6\text{S}_2)_3]^{4-}$ has an ϵ of approximately 18,700 (at 532 nm), while the iron-tris-ferrozine complex $[\text{Fe}(\text{C}_{20}\text{H}_{12}\text{N}_4\text{O}_6\text{S}_2)_3]^{4-}$ has an ϵ of approximately 22,000 (at 560 nm), the copper Chromazurol S complex $[\text{Cu}(\text{C}_{23}\text{H}_{13}\text{Cl}_2\text{O}_9\text{S})]^-$ has an ϵ of approximately 16,000 (at 522 nm), and the copper-bis-bathophenanthroline-disulfonic acid disodium complex $[\text{Cu}(\text{C}_{24}\text{H}_{16}\text{N}_2\text{O}_6\text{S}_2)_2]^{3-}$ has an ϵ of approximately 13,800 (at 481 nm).

Intensively colored organic pigments known in the prior art (typically large conjugated Π -systems) are, in principle, planar (e.g. Orange G). Due to this planarity, they have a disadvantageous high affinity, caused by the Van der Waals forces, for apolar surfaces such as the inner walls of the pipette tip, of the tubing, or of the well.

BRIEF DESCRIPTION OF THE DRAWINGS

An example of a molecule from the prior art is shown in Fig. 1 and two examples of metal complex pigments as provided herein for use in the method according to the present invention for determining the volume of the sample of a liquid are shown in Figs. 2 and 3.

- Fig. 1 shows Orange G
- Fig. 1a shows the structural formula
- Fig. 1b shows a horizontal projection, space-filling
- 5 Fig. 1c shows a side view, space-filling
- Fig. 2 shows copper(I)-bis-(bathophenanthroline-disulfonic acid disodium) complex
- Fig. 2a shows the structural formula
- 10 Fig. 2b shows a space-filling view
- Fig. 3 shows iron(II)-tris(ferrozine) complex
- Fig. 3a shows the structural formula
- 15 Fig. 3b shows a space-filling view

20 DETAILED DESCRIPTION OF THE INVENTION

Metal complex pigments provided according to the present invention have (in contrast to, for example, the prior art organic pigment Orange G) a three-dimensional, e.g. tetrahedral or octahedral, coordination geometry, which for steric reasons greatly hinders adsorption of this type of molecule to apolar surfaces. In addition, the ligands can be substituted with ionic groups such as sulfonic or carboxyl groups, which further amplifies the hydrophilic or lipophobic properties. Indicator ions in aqueous systems are very hydrophilic due to their charge and the spherical hydrate shell and therefore also do not tend to adsorb on apolar surfaces.

25

30

Adsorption tests with various complexes suggested according to the present invention have shown that no significant adsorption occurs on the walls of the pipetting needle or tubing.

- 5 It is desirable that the liquid properties relevant for pipetting be changed as little as possible during the measurement process. The addition of an indicator salt, which reacts in the well of a microplate with a chromogen ligand, to the pipetting solution only influences the liquid properties slightly due to its good solubility. Influence of the physical
10 properties is additionally reduced because the high extinction coefficient of the resulting complex permits the use of low initial concentrations of the indicator salt.

- At least a stoichiometric quantity of the chromogen ligands must be
15 present in the well before or after pipetting of the indicator salt solution. For reliable and rapid quantitative reaction, an excess of ligand can also be used. Any necessary buffer salts or redox active substances that convert the indicator ion into a suitable oxidation state are also present in the well. The actual pipetting procedure is therefore not influenced in
20 any way, which makes this measurement system widely variable.

- Most pigments are only suitable for a specific range of solvents due to their solubility. By complexing the indicator ion with a suitable auxiliary ligand, the indicator ion can be brought into solution in a suitable concentration in any desired solvent or mixture of solvents. For example,
25 iron(III) ions can be brought into solution in nonpolar solvents with 2,4-pentane dione as an $[\text{Fe}(\text{C}_5\text{H}_7\text{O}_2)_3]$ complex. A wide palette of derivatives is accessible from 2,4-pentane dione, from which the solubility of the iron complex in any desired solvent can be adjusted. In the well, an
30 auxiliary ligand is either quantitatively suppressed by a more chromogenic ligand and/or the complexed indicator ion is reduced by an oxidation number through a redox reaction, which allows the quantitative formation of a stronger complex with the chromogen ligand. Care

must be taken that the absorption spectrum of the auxiliary ligands does not overlap with that of the chromophoric complex.

- ELISA-Tests (ELISA = Enzyme-Linked Immuno Sorbent Assay) (cf. "PSCHYREMBEL Klinisches Wörterbuch" Walter de Gruyter GmbH & Co. KG, Berlin 1999, 258th edition) are now an integral part of clinical diagnostics and life science research. These tests frequently require one or more washing steps in the course of the test (cf. Lubert Stryer in "BIOCHEMISTRY", Freeman and Company, New York 1988, 3rd Edition, Page 63). In practice, the reaction liquid is suctioned from the coated microplates. Subsequently, buffer solutions or test reagents are dispensed into the wells. These two functions are conventionally performed by a microplate washer. In the first step, the device acts as a suction element, while in the second step, the device is used as a dispenser. New commercially available devices (such as those from TECAN Austria, Untersbergstrasse 1a, 5082 Groedig, Austria) can dispense several different buffer solutions, which can be used individually or together. In addition to standard, art-recognized criteria for dispensing, microplate washers must fulfill additional specifications in regard to the residual volume (e.g. 2 µl at most) after suctioning in a well.

- Microplates are preferably made of optically "perfect" materials. (If it were otherwise, positive absorption measurements would be obtained even with reagent "blank" solutions.) Microplates with flat bottoms and parallel walls are particularly preferably used. In microplates, particularly those with 384 or more wells, amplified meniscus formation can occur due to surface tension and liquid/wall interaction. If the menisci are irregular from well to well, different path lengths for the photometric measurements result, which negatively influences the reproducibility. Therefore, it is advisable to use microplates with low binding properties or otherwise modified surfaces to suppress the amplified meniscus formation.

- In a first exemplary embodiment of quantitative measurements provided according to the methods of the invention, the system "FeSO₄ in aqueous solution with FerroZine®" was used; "FerroZine®" is the registered trademark of Hach Company, P.O. Box 389, Loveland, CO 80539
- 5 USA. The samples were pipetted both in the single pipetting mode (12 single pipettings each) and in the multipipetting mode (12 aliquots). 20, 100, 200, or 1000 individual droplets, (intended droplet volume = 500 pl) respectively, were dispensed.
- 10 An aqueous 0.25 M FeSO₄ solution with FerroZine® and ammonium acetate buffer was used for producing a calibration curve. The resulting complex solution was stabilized with ascorbic acid. From this initial solution, measurement solutions were produced through dilutions that corresponded to pipetting volumes of 2.5 nl, 5.0 nl, 10.0 nl, 20.0 nl, 40.0
- 15 nl, and 80.0 nl in 200 µl. Twelve 200 µl aliquots of each of these measurement solutions were pipetted by hand into a microplate and the optical absorption and/or the optical densities (OD) were measured with a microplate photometry reader. The calibration curve was calculated through the measurement points by means of linear regression.
- 20 For the volume determinations, 100 µl of a 3.25 mM FerroZine® solution with ascorbic acid, buffered with ammonium acetate, was placed in the wells of a microplate. Ten nanoliters and 50 nl of a 0.25 M FeSO₄ solution stabilized with ascorbic acid was pipetted into this with a pipetting robot. The pipettings of 100 nl and 500 nl were performed with a
- 25 0.025 M FeSO₄ solution stabilized with ascorbic acid.

After pipetting, the volume was "topped up" to a total volume/well of 200 µl with demineralized water in the individual wells and the solutions

30 were mixed well in the microplates by mechanical shaking. The optical absorption of the colored complex solution in the wells of a microplate was then measured in a microplate photometry reader and the volumes were calculated with reference to the calibration curve.

The results achieved with the system "FeSO₄ in aqueous solution with FerroZine[®]" are shown in the following tables 1 and 2:

5 Table 1

Single Pipetting Mode			
Intended volume	Average volume of the 12 single pipettings	ACC	CV
10 nL	9.7 nL	3.0%	2.9 %
50 nL	48.0 nL	4.0%	1.2 %
100 nL	101.8 nL	1.8%	1.5 %
500 nL	497.5 nL	0.5%	1.5 %

10 Table 2

Multipipetting Mode			
Intended volume	Average volume of the 12 aliquots	ACC of the aliquots	CV of the aliquots
10 nL	9.8 nL	2.0%	1.4 %
50 nL	48.1 nL	3.8%	2.5 %
100 nL	99.3 nL	0.7%	4.0 %
500 nL	509.0 nL	1.8%	2.8 %

In a second exemplary embodiment of quantitative measurements obtained according to the methods of the invention, the system "iron-tris(acetyl acetate) in 100% dimethyl sulfoxide (DMSO) with FerroZine[®]" was used. The samples were pipetted both in the single pipetting mode (12 single pipettings each) and in the multipipetting mode (12 aliquots). Individual droplets (numbering 20, 100, 200, or 1000 individual droplets, respectively, with an intended droplet volume = 400 pL)

were dispensed. A 0.063 M iron-tris(acetyl acetonate) solution in pure DMSO was used for the calibration curve. From this initial solution, measurement solutions were produced, through dilutions with ammonium acetate buffer, ascorbic acid, and FerroZine®, which corresponded to pipetting volumes of 2.5 nl, 5.0 nl, 10.0 nl, 20.0 nl, 40.0 nl, and 80.0 nl in 200 µl. Twelve 200 µl aliquots of each of these measurement solutions were pipetted by hand into a microplate and the optical absorption and/or the optical densities (OD) were measured with a microplate photometry reader. The calibration curve was calculated through the measurement points by means of linear regression. For the volume determinations, 100 µl of a 3.25 mM FerroZine® solution with ascorbic acid buffered with ammonium acetate was placed in each of the wells of a microplate. Aliquots (8 nl, 40 nl, 80 nl, and 400 nl) of a 0.063 M iron-tris(acetyl acetonate) solution in pure DMSO were pipetted into this solution with the pipettor.

After pipetting, the volume was "topped up" to a total volume of 200 µl/well with demineralized water in the individual wells and the solutions were mixed well in the microplates by mechanical shaking. The optical absorption of the colored complex solution in the wells of the microplate was then measured in a microplate photometry reader and the volumes were calculated with reference to the calibration curve. The results achieved with the system "iron-tris(acetyl acetonate) in 100% dimethyl sulfoxide (DMSO) with FerroZine®" are shown in the following tables 3 and 4:

Table 3

Single Pipetting Mode			
Intended volume	Average volume of the 12 single pipettings	ACC	CV
8 nl	8.3 nl	3.8%	1.7%
40 nl	37.8 nl	5.5%	2.6%

80 nl	71.2 nl	11.0%	1.1%
400 nl	356.9 nl	10.8%	1.8%

Table 4

Multipipetting Mode			
Intended volume	Average volume of the 12 aliquots	ACC of the aliquots	CV of the aliquots
8 nl	8.0 nl	0.0%	1.2%
40 nl	38.1 nl	4.8%	1.0%
80 nl	75.8 nl	5.3%	0.8%
400 nl	377.9 nl	5.5%	1.1%

5

As these examples show, the methods of the invention provide a way to accurately and reproducibly dispense varying small amounts of liquid, and to have confidence in the amount dispensed. The method provided by the present invention actually permits the volume of a sample of a liquid to be determined and calibrated in the sub-microliter range, using the metal complex pigments and devices provided herein.

The present invention can also be used to determine the volume of a sample of a liquid and calibration in the sub-microliter range if anions are used as the indicator to stain the liquid (A). Complexing with a specific ligand also generates the staining of the sample in these cases. Examples of ligands for complexing of F^- , Cl^- and/or $H_2PO_4^-$ ions in (dichloromethane) are described in the article by Miyamji *et al.* (2000, *Angew. Chem.* 112: 1847 - 1849): anthraquinone functionalized systems covalently bonded at the β position, particularly calix[4]pyrrole-anthraquinone, have been shown to be very sensitive sensors for detecting these anions.

25